AWARD NUMBER: W81XWH-16-2-0050

TITLE: The Role of Desert-Dust Metals in the Pathobiology of Gulf War Illness

PRINCIPAL INVESTIGATOR: John F. Kalinich, PhD

RECIPIENT: Henry M. Jackson Foundation for the Advancement of Military Medicine

Bethesda, MD 20817

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE
The Role of Desert-Dust Metals in the Pathobiology of Gulf War Illness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2-0050

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John F. Kalinich, PhD

E-Mail: john.kalinich@usuhs.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Henry M. Jackson Foundation for the Advancement of Military Medicine
6720A Rockledge Drive
Bethesda, MD 20817

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Report contains color photos.

14. ABSTRACT
After the First Persian Gulf War (1990-1991), many U.S. personnel reported suffering from a chronic multi-symptom disease eventually called "Gulf War Illness". We hypothesize that exposures to pyridostigmine bromide, permethrin, and/or DEET adversely affect the permeability of the blood-brain barrier allowing metals solubilized from inhaled desert dust particles to enter the brain. As a consequence, normal metal homeostasis is disrupted resulting in extensive oxidative damage and neurological dysfunction. In Year 1 of this 3 year project we have established and characterized the human cell lines that comprise the blood brain barrier. In addition, toxicity assessments of pyridostigmine bromide, permethrin, DEET, aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc on these cell lines have been completed. In Year 2, we will assess the effect of these compounds on blood-brain barrier permeability and induction of oxidative damage and inflammation.

15. SUBJECT TERMS
Gulf War Illness, desert dust, metals, DEET, permethrin, pyridostigmine bromide

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	29	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In 1990-1991, the United States and its Coalition allies responded militarily to the Iraqi invasion of Kuwait. Close to 700,000 U.S. military personnel served in the resulting Persian Gulf War. Soon afterward, many of these individuals reported suffering from a chronic multi-symptom disease that was given the moniker "Gulf War Illness." For the past 25 years investigators have searched for a cause for these ailments, but as yet no definitive cause has been identified. The hypothesis of this research is that combined exposures to pyridostigmine bromide (PB), permethrin (PM), and/or DEET adversely affect the permeability of the blood-brain barrier (BBB) allowing metals solubilized from inhaled desert dust particles to enter the brain. As a consequence, normal metal homeostasis is disrupted resulting in extensive oxidative damage and neurological dysfunction. This project uses commercially available human brain microvascular endothelial cells and astrocytes in an *in vitro* blood-brain barrier model system to assess the effects of PB, PM, DEET, and their metabolites on BBB permeability. In addition, those compound(s) that affect BBB permeability will be further tested for their ability to enhance the translocation of metals across the BBB.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Gulf War Illness, desert dust, metals, DEET, permethrin, pyridostigmine bromide

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Listed below are the five major task area associated with this project, start and end dates, and percentage of completion.

Major Task 1 – Experimental Preparation, Year 1/Month 1 to Year 1/Month 5, 100% completed.

Major Task 2 – Assessment of Cell Viability after Test Compound Exposures, Year 1/Month 6 to Year 1/Month 10, 100% completed.

Major Task 3 – Determination of BBB Permeability Changes after Administration of Test Compounds, Year 1/Month 11 to Year 2/Month 5, 5% completed.

Major Task 4 – Determination of Indicators of Oxidative Stress and Inflammation in BBB Cells after Exposure to Test Compounds, Year 2/Month 6 to Year 3/Month 2, 0% completed.

Major Task 5 – Data Compilation, Statistical Analysis, and Preparation of Final, Year 3/Month 3 to Year 3/Month 12, 0% completed.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

In Year 1 of this project, financial accounts were established, personnel hired and trained, and all required regulatory approvals were obtained. Also in Year 1, our laboratory was extremely fortunate to be joined by Dr. Jessica Hoffman (Federal Government (GS) Employee), whose expertise in neurobiology and primary cell culture will greatly enhance the probability of success for this project. After numerous delays by one of the vendors, we were finally able to secure the human astrocyte and endothelial cells required for the project. The cell lines were successfully established in our laboratory and toxicity assessments of pyridostigmine bromide, permethrin, and DEET, as well as their metabolites (i.e., 3-hydroxy-1-methylpyridinium bromide; 3-phenoxybenzyl alcohol; 3-phenoxybenzoic acid; N,N-diethyl-*m*-hydroxymethylbenzamide; and N-ethyl-*m*-toluamide) completed. The toxicity assessments included the following tests for cell viability and function: MTT conversion, Neutral Red uptake, and lactate dehydrogenase release. In addition, changes in morphology were determined microscopically. The toxicity of the metals to be tested in this project was also determined using the aforementioned tests. Metals tested were those found in elevated levels in the desert dust of Iraq and Kuwait and included: aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc. The viability assay procedures and experimental results can be found in the Appendices. From these studies, non-toxic concentrations of all test components were selected for further study. These results can be found in the Appendices, but generally were in the range of 1-10 µM for the organic compounds and 1 µM for the metals. We have also successfully validated the analytical methods for our test metal using Inductively Coupled Plasma-Mass Spectrometry.

Also in Year 1, the Dynamic In Vitro Blood Brain Barrier (DIV-BBB) System from FloCel was purchased. We spent a great deal of time trying to establish the system and determine its functional characteristics. We found that even after numerous conversations with FloCel technical support we were unable to get the system to perform as advertised by FloCel. As a result, a decision was made to switch to our back-up plan of using multi-cultured transwell inserts to determine BBB permeability after test compound exposures. However, work in this area has been slowed due to an inexplicable delay in obtaining the astrocyte culture medium from Lonza. After numerous promised delivery dates were missed, we have now abandoned trying to obtain the Lonza medium and have switched to a secondary vendor. This switch has necessitated retesting our astrocyte culture to determine that their functionality is comparable to that seen in the previously used medium. More on these setbacks and troubleshooting efforts can be found in Section 5.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period we will optimize the transwell co-culture BBB model and complete the characterization of blood brain barrier permeability changes after administration of test compounds (pyridostigmine bromide, permethrin, DEET, aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc). In addition, we will initiate a study to determine the indicators of oxidative stress and inflammation in blood brain barrier cells after exposure to test compounds

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The FloCel system failed to function as a 3D co-culture BBB model as advertised, despite a full array of troubleshooting efforts and numerous consultations with FloCel technical support, so we have moved to our back-up model system that utilizes a transwell co-culture system. There is no change in the project objectives or scope of the research with this change.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

During the initial start-up of this project we had several vendor-associated issues, in addition to the FloCel issue described above. First, the recommended vendor (ScienCell, Carlsbad, CA) for the human brain microvascular endothelial cells informed us that this cell line was backordered for at least 6 to 12 months. We eventually secured the cells from another vendor (Cell Systems, Kirkland, WA). Later in Year 1, the vendor (Lonza, Walkersville, MD) supplying the medium for the human astrocyte cell line informed us that they would be unable to provide the required medium for the foreseeable future. We then switched vendors to Gibco (Grand Island, NY). This change necessitated reassessing growth characteristics of the human astrocyte cell line. This was completed and we remain on schedule.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Jessica F. Hoffman, Christine E. Kasper, and John F. Kalinich. Effect of Gulf War "Desert-Dust" compounds on the viability and permeability of the blood- brain barrier in a 3D model. Abstract submitted Society for Neuroscience meeting (11-15 November 2017, Washington, DC).

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: John Kalinich, PhD
Project Role: Team Leader
Researcher Identifier: 0000-0003-1591-9389
Nearest person month worked: 2
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: Federal Government Employee (Department of Defense)

Name: Christine Kasper, PhD RN, FAAN FACS
Project Role: Co-investigator,
Research Identifier: 0000-0002-7784-2519
Nearest person month worked: 1
Contribution to Project: Responsible for experimental planning
Funding Support: Federal Government Employee (Department of Veterans Affairs)

Name: Vernieda Vergara, BS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for cell culture maintenance and metal analysis.

Name: Jessica Hoffman, PhD
Project Role: Co-investigator
Researcher Identifier: 0000-0003-1858-8394
Nearest person month worked: 5
Contribution to Project: Responsible for establishment of cell model systems and testing.
Funding Support: Federal Government Employee (Department of Defense)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not Applicable.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Not required.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

APPENDICES

Effect of Gulf War "Desert-Dust" compounds on the viability and permeability of the blood- brain barrier in a 3D model

Jessica F. Hoffman[1], Christine E. Kasper[2], and John F. Kalinich[1]
[1]Internal Contamination and Metal Toxicity Program, Armed Forces Radiobiology Research Institute, Uniformed Services University, Bethesda, MD
[2] Daniel K. Inouye Graduate School of Nursing, Uniformed Services University, Bethesda, MD

Gulf War Illness (GWI) refers to the chronic multi-symptom illness characterized by cognitive problems, fatigue, and muscle pain suffered by over one-third of American veterans who served in the Persian Gulf War in 1990-1991. Investigations into potential causes suggest a multiple exposure scenario, possibly to a combination of the nerve gas prophylactic pyridostigmine bromide (PB), insecticide N,N-diethyl-*m*-toluamine (DEET), and pesticide permethrin (PM), rather than a single exposure incident. Experiments have shown combining exposures to PB with PM or DEET disrupts the blood-brain barrier (BBB) and causes neurological and behavioral deficits, but does not account for all reported symptoms of GWI. Another study suggests PB alone can increase BBB permeability, allowing a virus to cross over into the brain where it is not normally found. Other studies suggest that respiratory exposure to the fine-grained sand particles found in the area, deemed "desert dust," could also be linked to GWI. Analysis of this desert dust found high levels of a variety of metals, including aluminum, iron, uranium, nickel, cobalt, copper, strontium, manganese, and zinc. Under certain conditions, metals have been known to cross the BBB into the brain and induce neuronal injury and behavioral changes.

We hypothesize that combined exposures of PB, PM, and DEET adversely affect BBB permeability, allowing metals solubilized from inhaled desert dust particles to enter the brain. To test this, we used a combination of traditional cell culture and a 3D dynamic *in vitro* model of the BBB (DIV-BBB). First, human brain endothelial cells (Cell Systems, ACBRI 376) and human astrocytes (Lonza, CC-2565) were individually exposed to PB, PM, DEET, nickel, cobalt, strontium, zinc, manganese, copper, iron, aluminum, or depleted uranium (dose range 0.1 to 1000 µM) and assessed for viability and function using standard cell culture techniques. For most compounds, we found 1 µM to be a sufficiently sub-toxic dose, which was then used in the 3D DIV-BBB system (FloCel). Endothelial cells in the luminal (blood) side of a 3D cartridge were co-cultured with astrocytes in the abluminal (brain) side of the cartridge, and allowed to establish tight junctions evidenced by a high transendothelial electrical resistance (TEER) value. The closed system, which provided a more realistic model of blood flow and metabolite exchange across a BBB, was exposed to sub-toxic levels of PB, PM, and DEET for 24 hours, followed by sub-toxic levels of each metal and monitored for changes in TEER and the translocation of metal from the luminal to abluminal side.

Funding: CDMRP Gulf War Illness Research Program: W81XWH-16-2-0050

Cell Viability Assays

Lactate Dehydrogenase (LDH) Release

Plasma membrane integrity was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI). The assay for plasma membrane integrity is based on the quantitative colorimetric determination of lactate dehydrogenase (LDH). LDH, a stable cytosolic enzyme, is released into the culture medium when damage occurs to the plasma membrane of the cell. The assay was conducted as follows. Cells were plated in 96-well tissue culture plates at a predetermined concentration to assure maximum response for the assay and treated with varying concentrations of test compounds for 24 h at 37ºC. The plates were then centrifuged at 250 x g for 4 min. An aliquot (50 µl) of the resulting supernatants was then added to the wells of a fresh 96-well plate. "Substrate Mix" (50 µl, supplied in the kit) was added to each well and the plate incubated for 30 min in the dark at room temperature. The color reaction was terminated by the addition of 50 µl of "Stop Solution" (supplied in the kit) and the absorbance of the reaction mixtures determined at 490 nm in a microplate reader (SpectaMax Model 250 Microplate Spectrophotometer, Molecular Devices Corporation, Sunnyvale, CA). LDH release from treated cells was compared to untreated cells.

Note: We found no difference in LDH release between control and treated cells suggesting that treatment did not result in plasma membrane damage.

Metabolic Viability Assessment

Metabolic viability (MTT assay) was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation). The assay for metabolic viability is based upon the ability of dehydrogenase enzyme systems, located in the cell mitochondria, to reduce a tetrazolium compound to a colored formazan product. The water soluble nature of this product allows it to be easily detected colorimetrically. The assay was conducted as follows. Cells were plated in 96-well tissue culture plates at a predetermined concentration to assure maximum response for the assay. After allowing time for the cells to adhere to the plate, appropriate concentrations of the test compounds were added and the plates returned to the incubator for 24 h. One hour prior to termination of the incubation period, 10 µl of CellTiter 96® Aqueous One Solution Reagent was added to each well of the plate and the plate returned to the incubator for 1 h. After this time, the absorbance was determined at 490 nm using a microplate reader. Metabolic viability of the treated cells was compared to untreated control cells.

Cell Function Assay

The Neutral Red (NR) Assay measures the ability of viable cells to take up and concentrate neutral red into lysosomes. Non-viable cells do not take up the dye. Cells were plated in 96-well tissue culture plates at a predetermined concentration to assure maximum response for the assay. After allowing time for the cells to adhere to the plate, appropriate concentrations of the test compounds were added and the plates returned to the incubator for 24 h. Two hours before the end of the incubation period, the neutral red dye solution (Sigma Chemical) was added to the cells. At the end of the incubation period, the cells were washed and then solubilized to release the internalized neutral red. The amount of dye was then determined at 540 nm using a microplate reader.

Abbreviations Used in Figures

Al	Aluminum
Co	Cobalt
Cu	Copper
DEET	N,N-Diethyl-*m*-toluamide
DEET-ET	N-ethyl-*m*-toluamide
DEET-OH	N,N-diethyl-*m*-hydroxymethylbenzamide
Di-PB	3-Hydroxy-1-methylpyridinium bromide
DMSO	Dimethylsulfoxide
DU	Depleted uranium
Fe	Iron
LDH	Lactate dehydrogenase
Mn	Manganese
Ni	Nickel
NR	Neutral Red
PB	Pyridostigmine bromide
PM	Permethrin
PM-acid	3-Phenoxybenzoic acid
PM-alcohol	3-Phenoxybenzyl alcohol
Sr	Strontium
Zn	Zinc

Figure 1: Human Brain Microvascular Endothelial Cells – Metal Toxicity Curves (Neutral Red Assay)

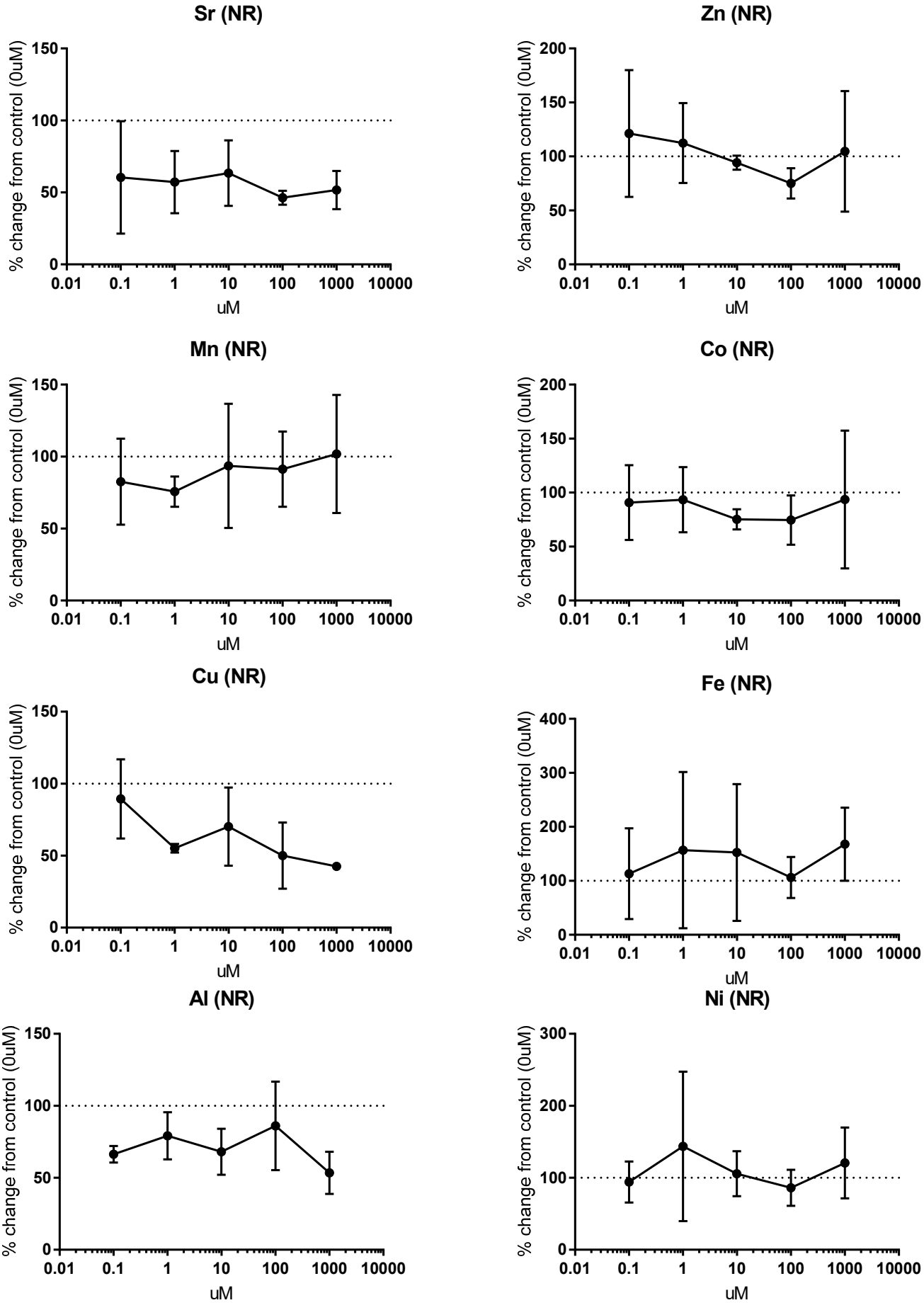
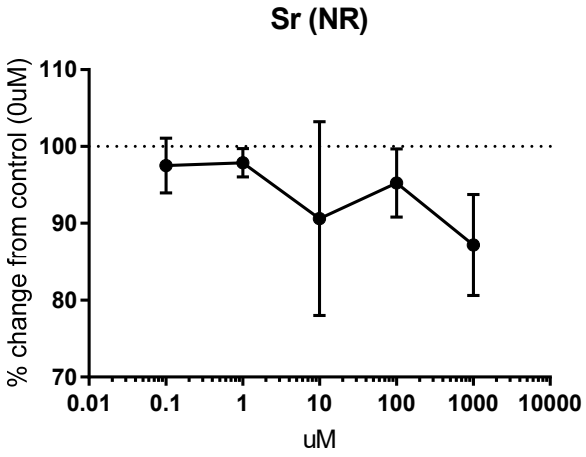
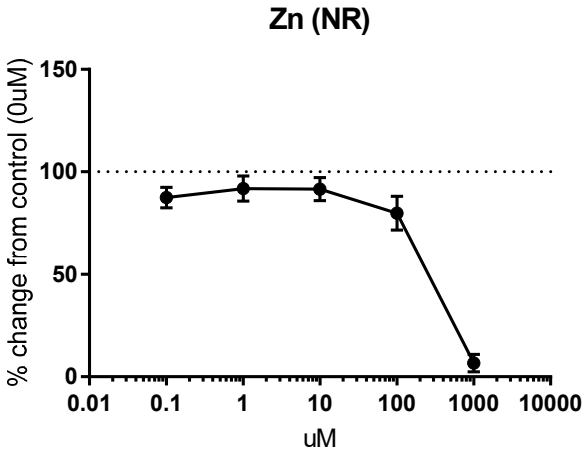
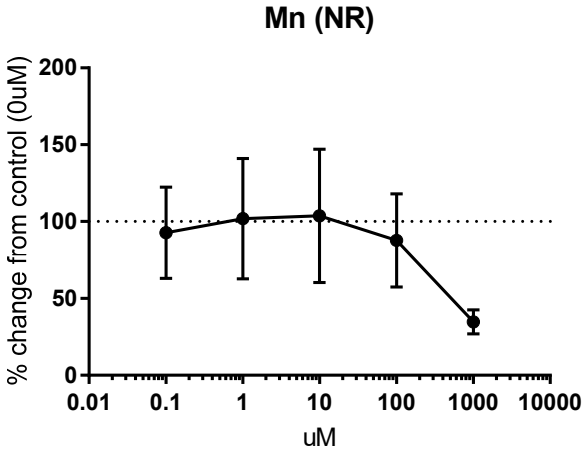
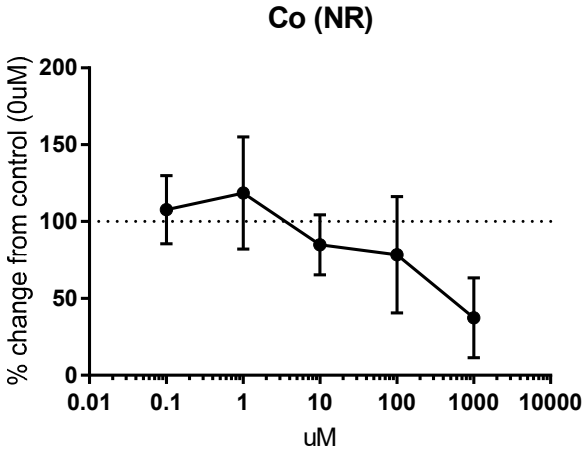


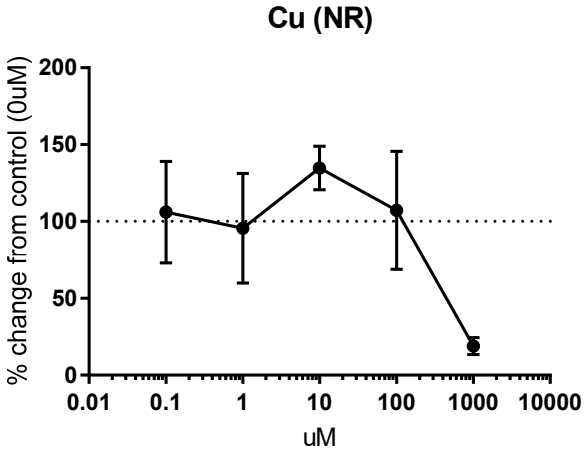
Figure 2: Human Astrocytes – Metal Toxicity Curves (Neutral Red Assay)

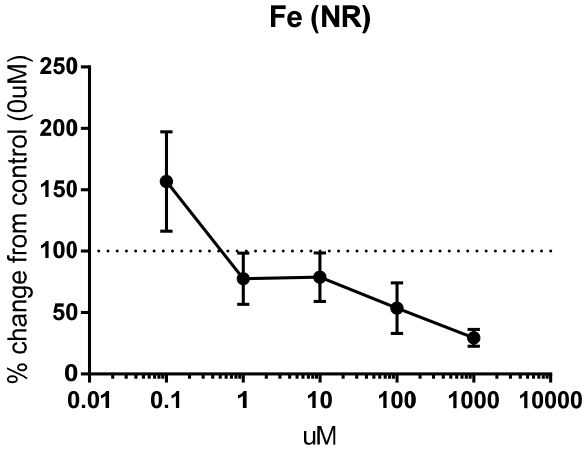


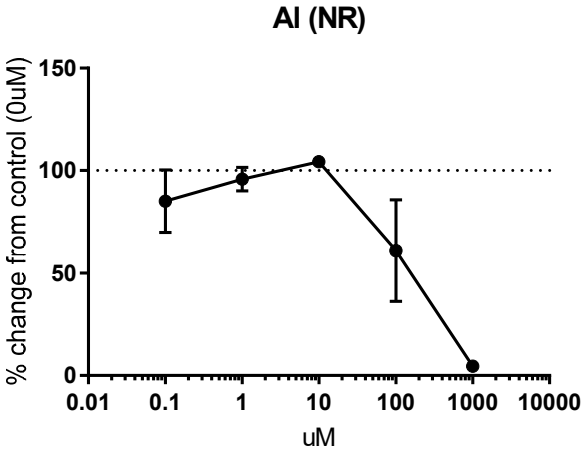












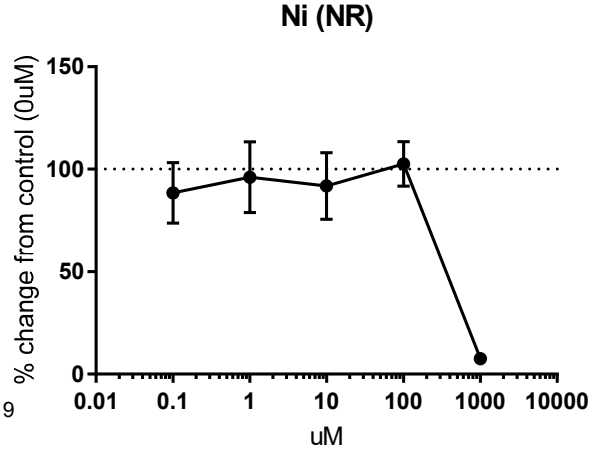
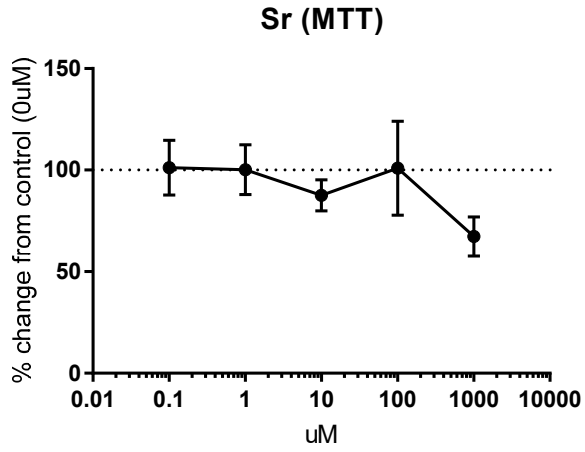
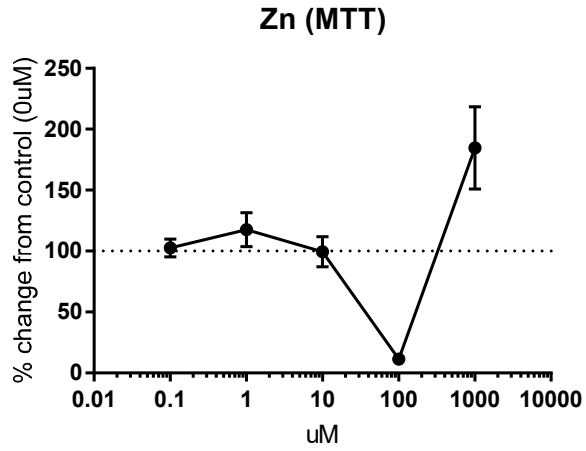
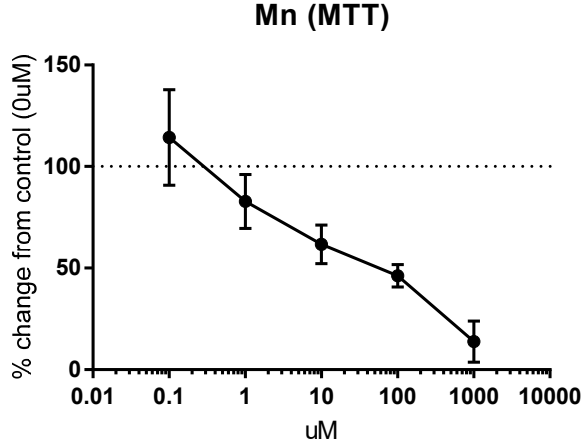
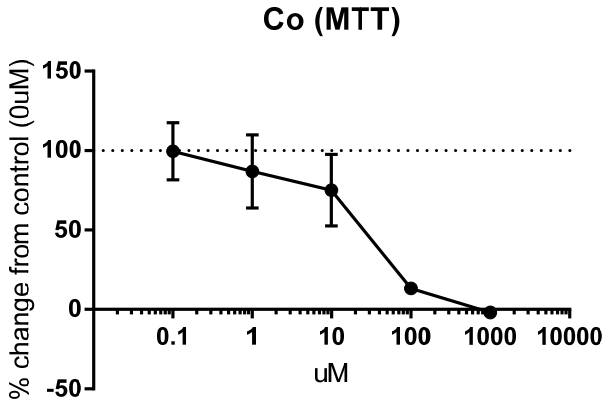


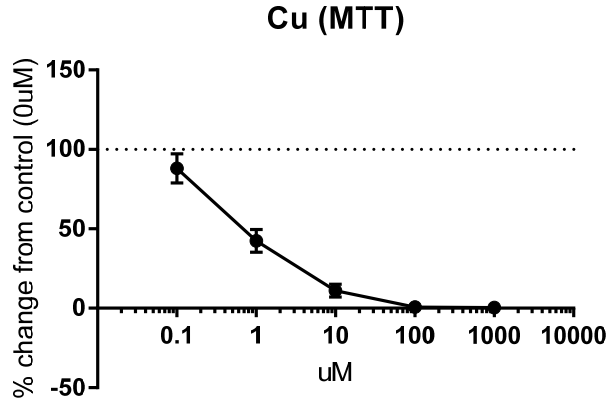
Figure 3: Human Brain Microvascular Endothelial Cells – Metal Toxicity Curves (MTT Assay)

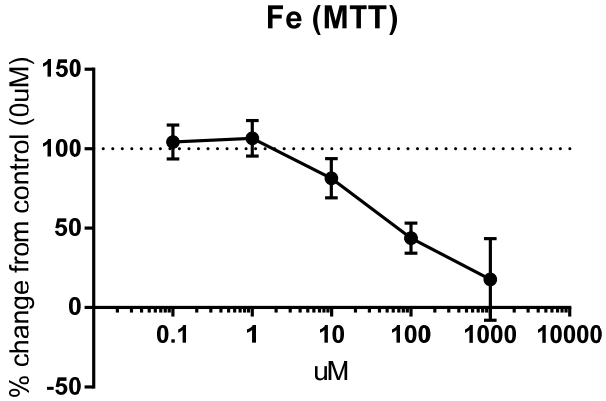


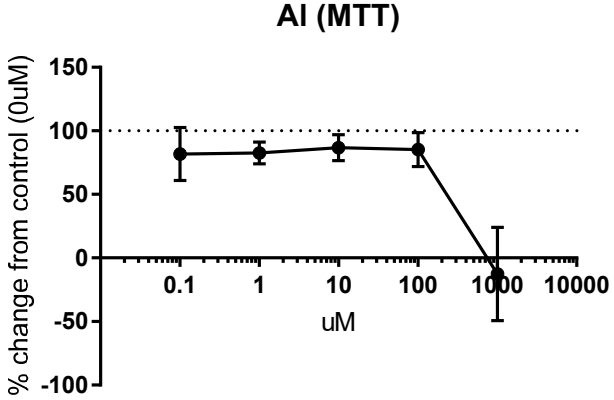












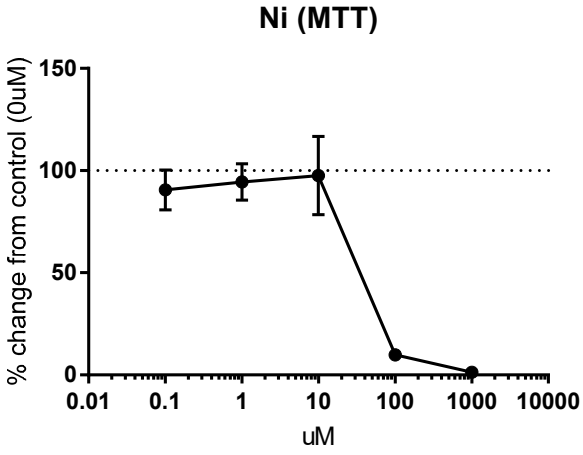
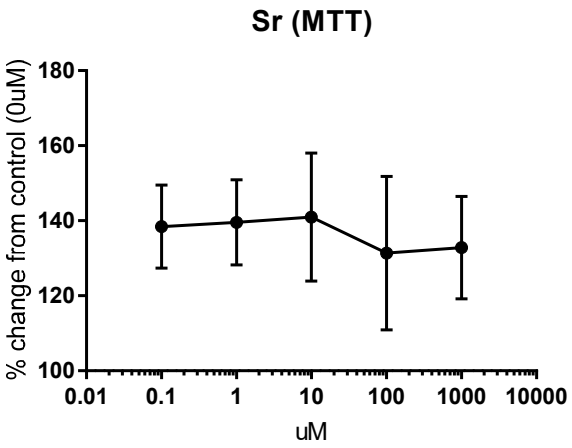
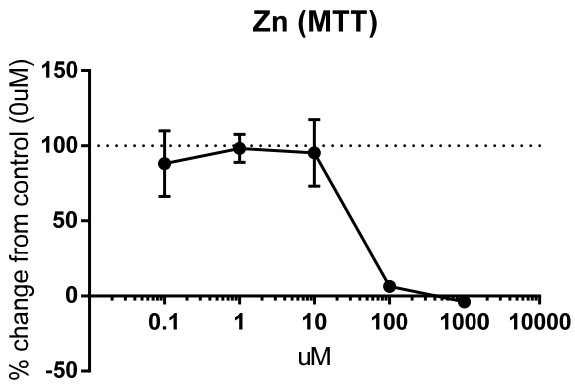
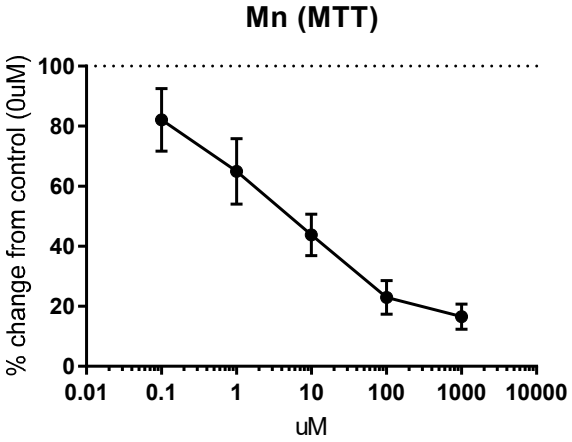
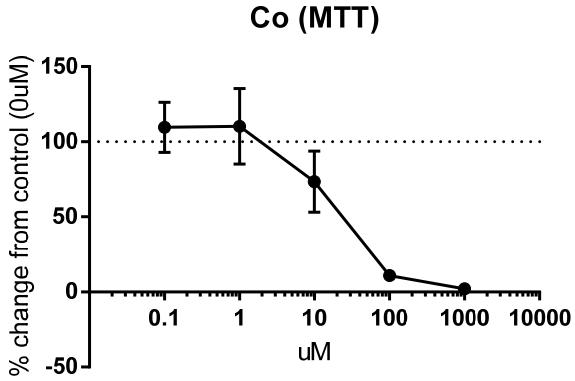


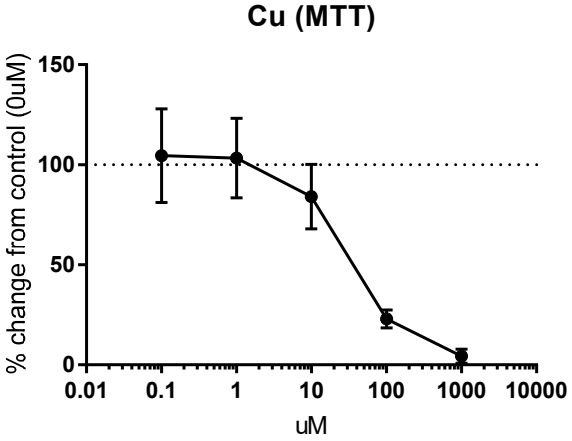
Figure 4: Human Astrocytes – Metal Toxicity Curves (MTT Assay)

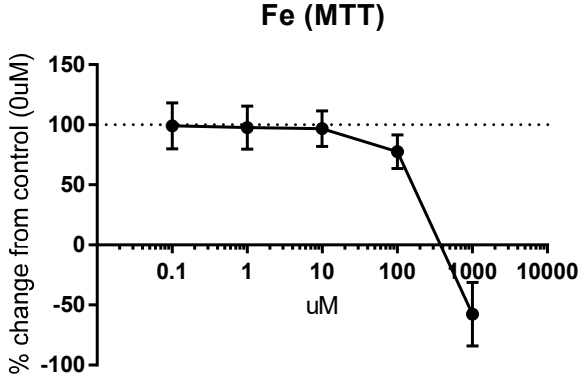


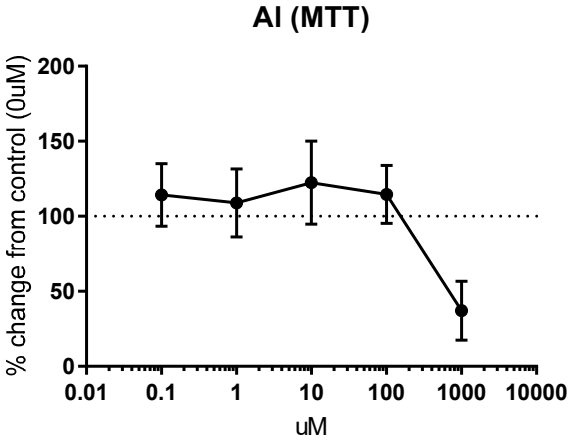












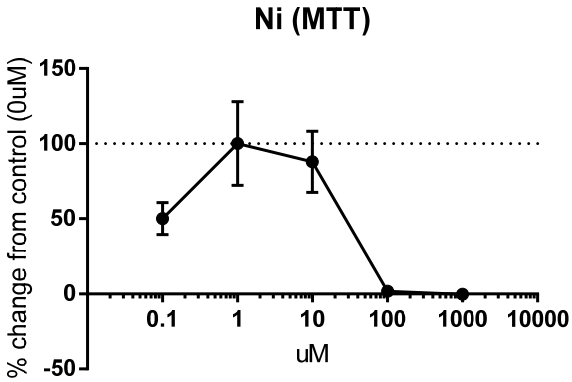
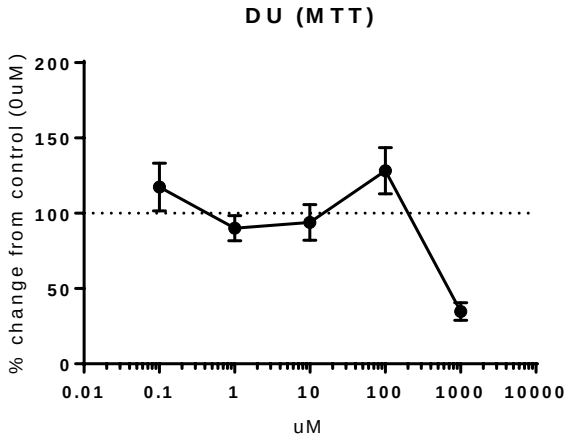
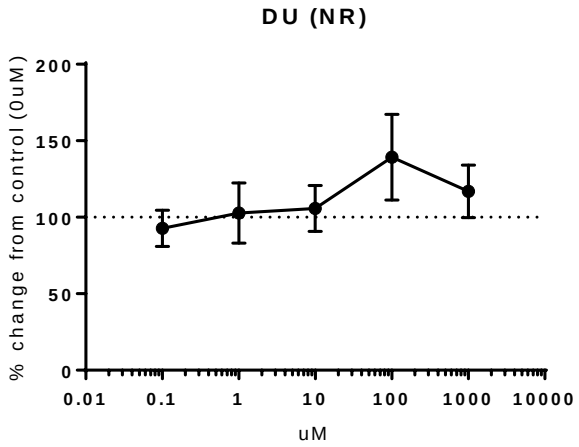


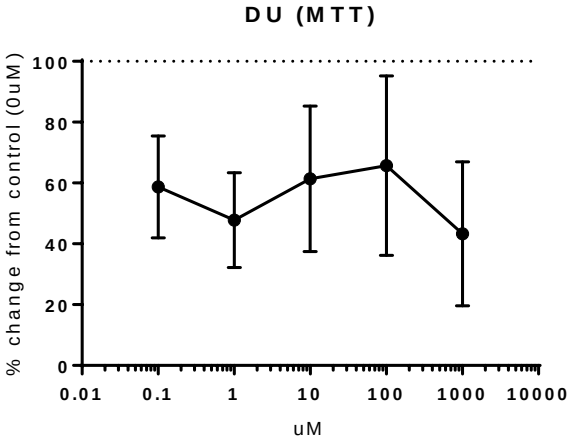
Figure 5: Depleted Uranium Toxicity Curves

Endothelial Cells





Astrocytes



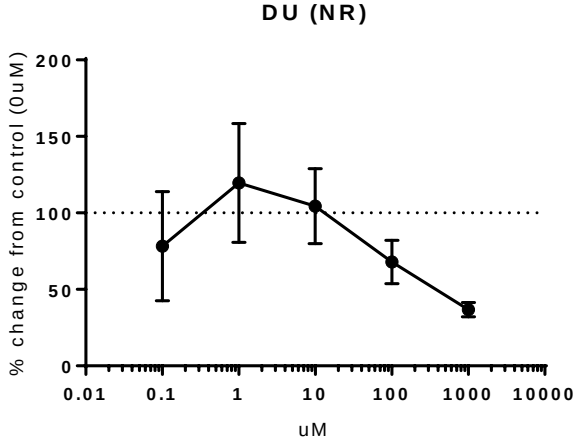
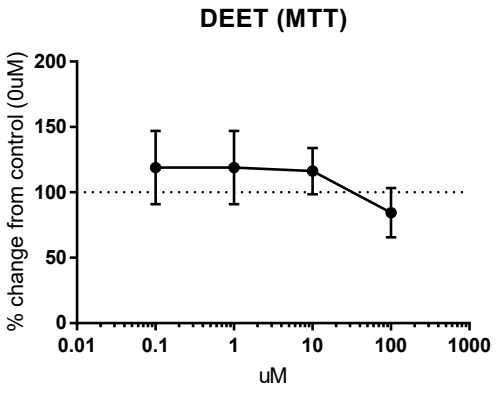
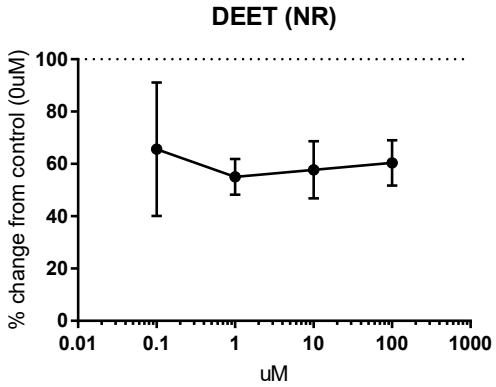
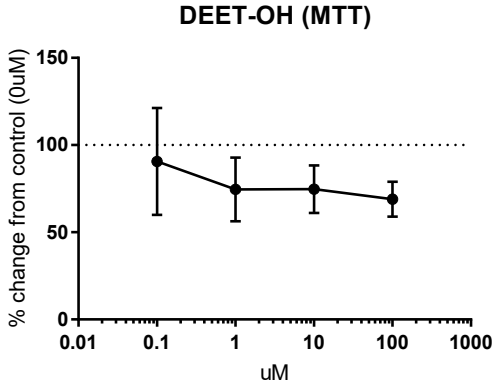
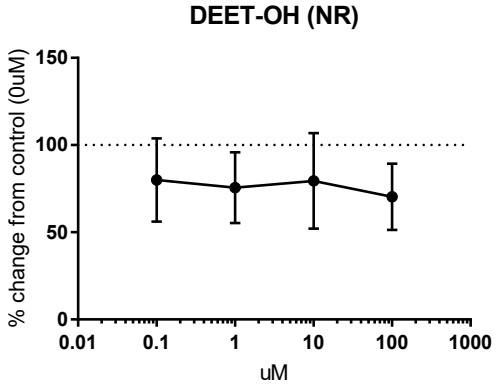


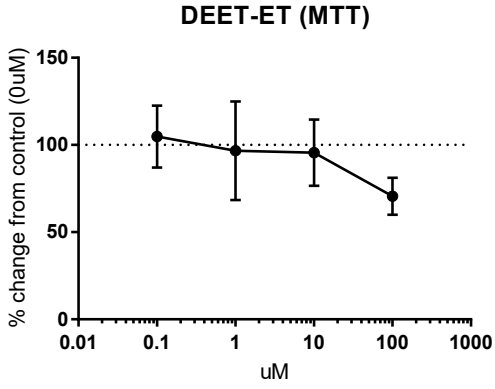
Figure 6: Human Brain Microvascular Endothelial Cells – Organic Toxicity Curves

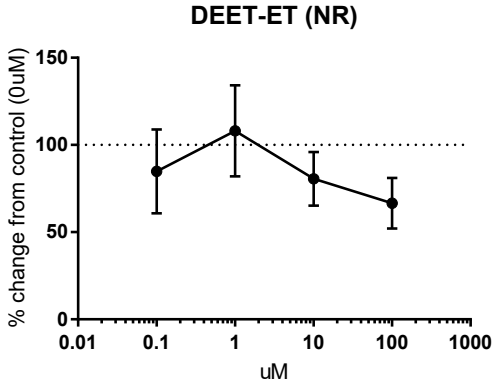


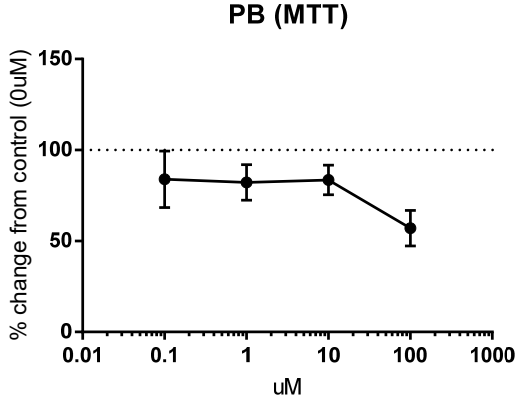












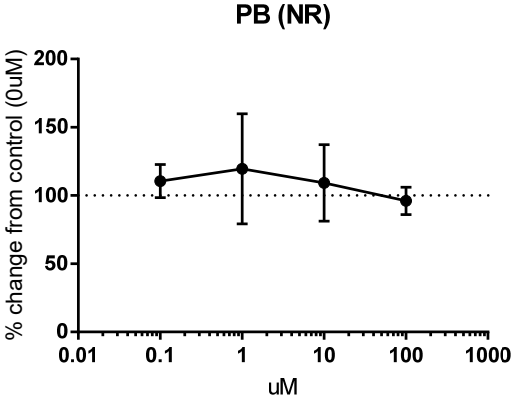


Figure 7: Human Brain Microvascular Endothelial Cells – Organic Toxicity Curves

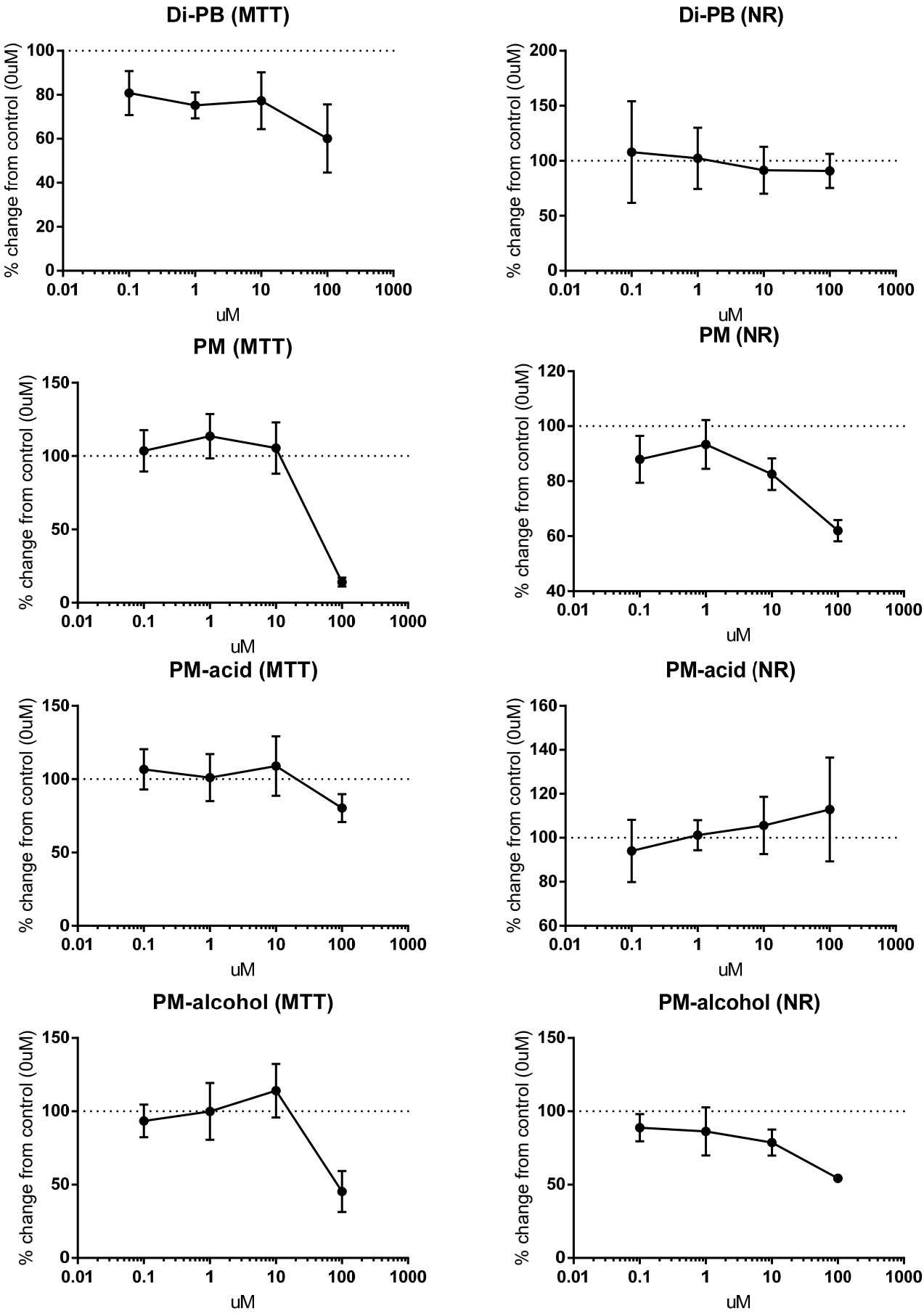


Figure 8: Human Astrocytes – Organic Toxicity Curves (Set 1)

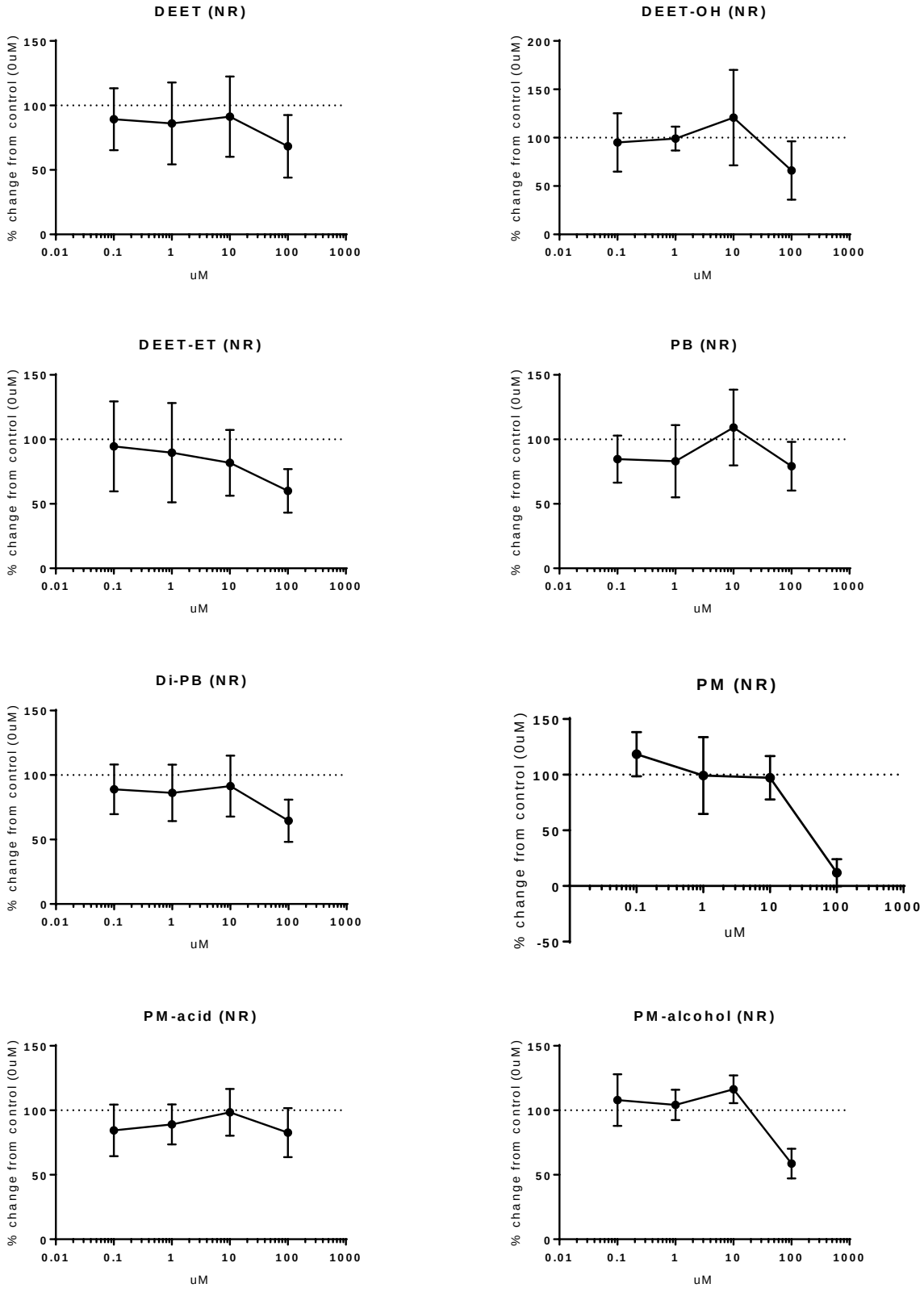
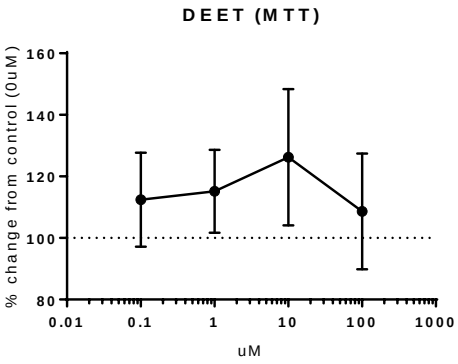
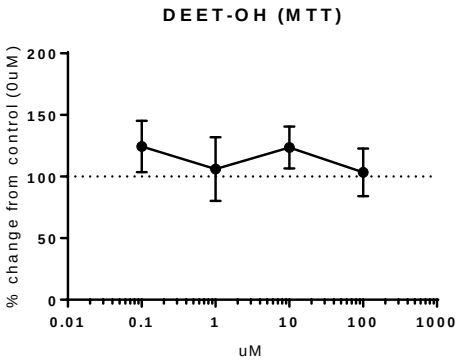
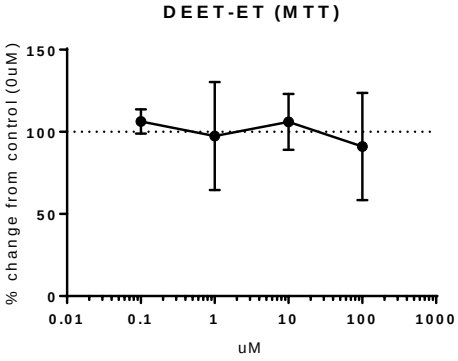
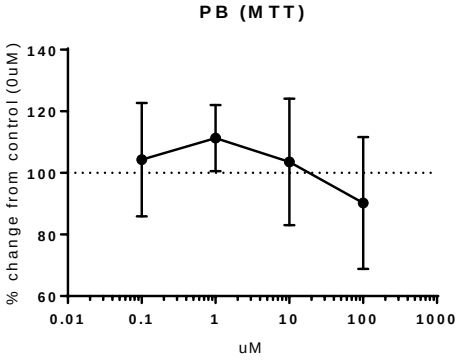


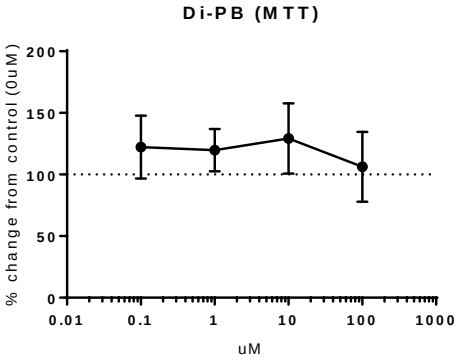
Figure 9: Human Astrocytes – Organic Toxicity Curves (Set 2)

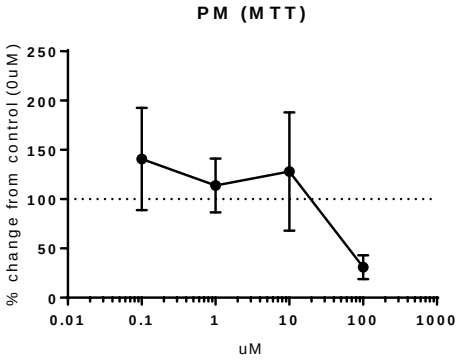


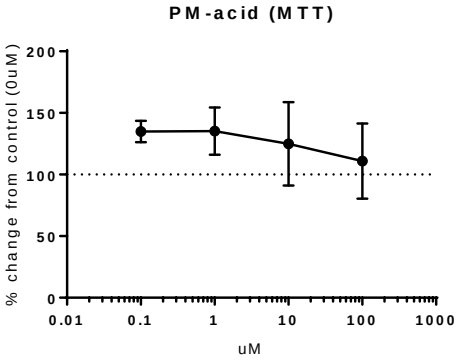












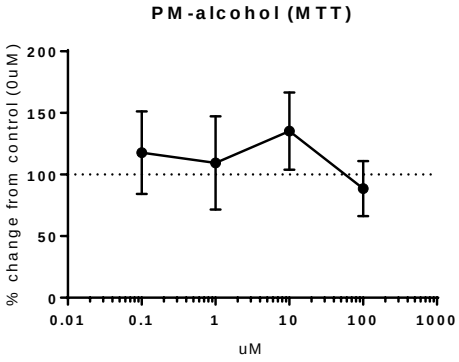


Figure 10: Human Brain Microvascular Endothelial Cells (10X)

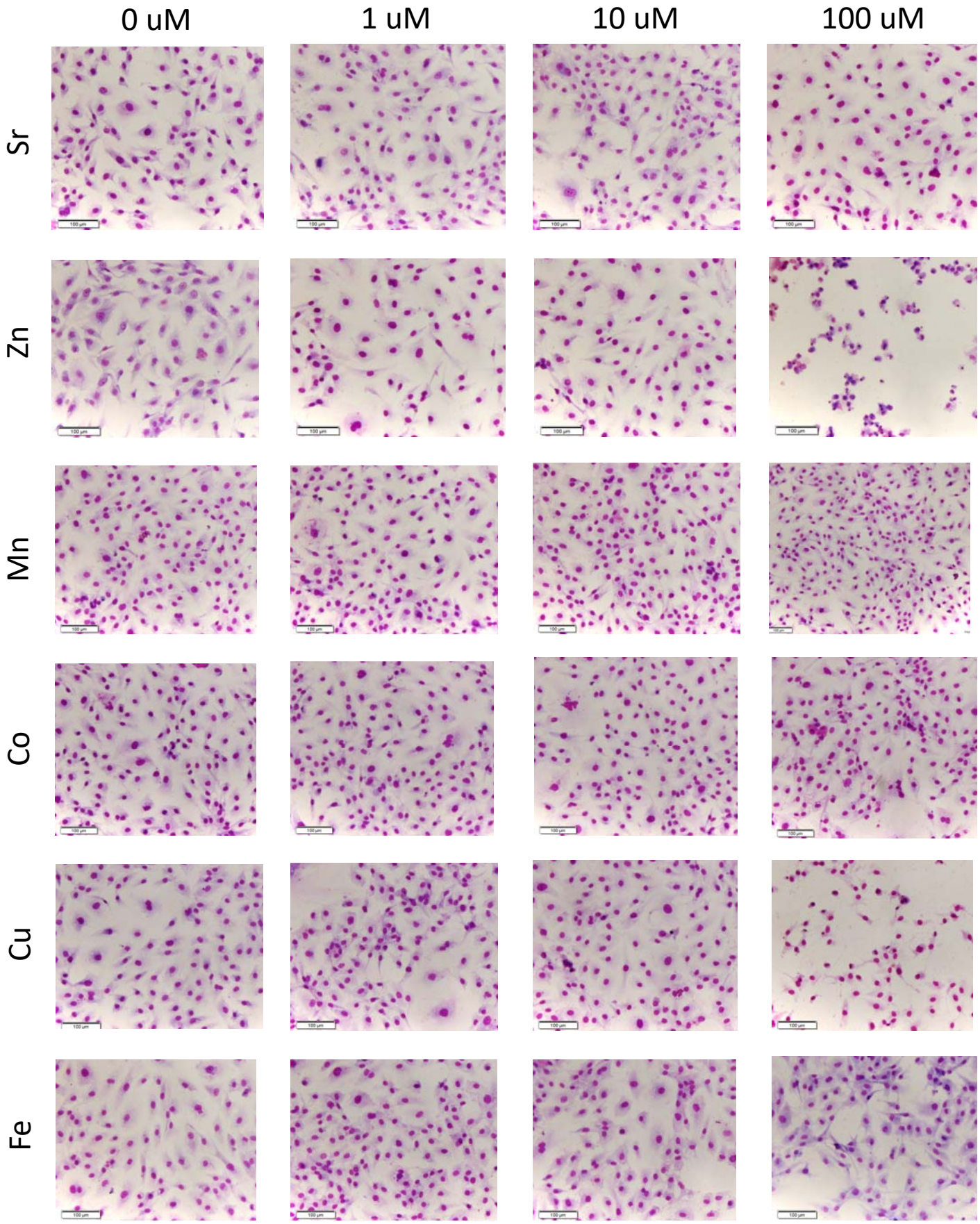


Figure 11: Human Brain Microvascular Endothelial Cells (10X)

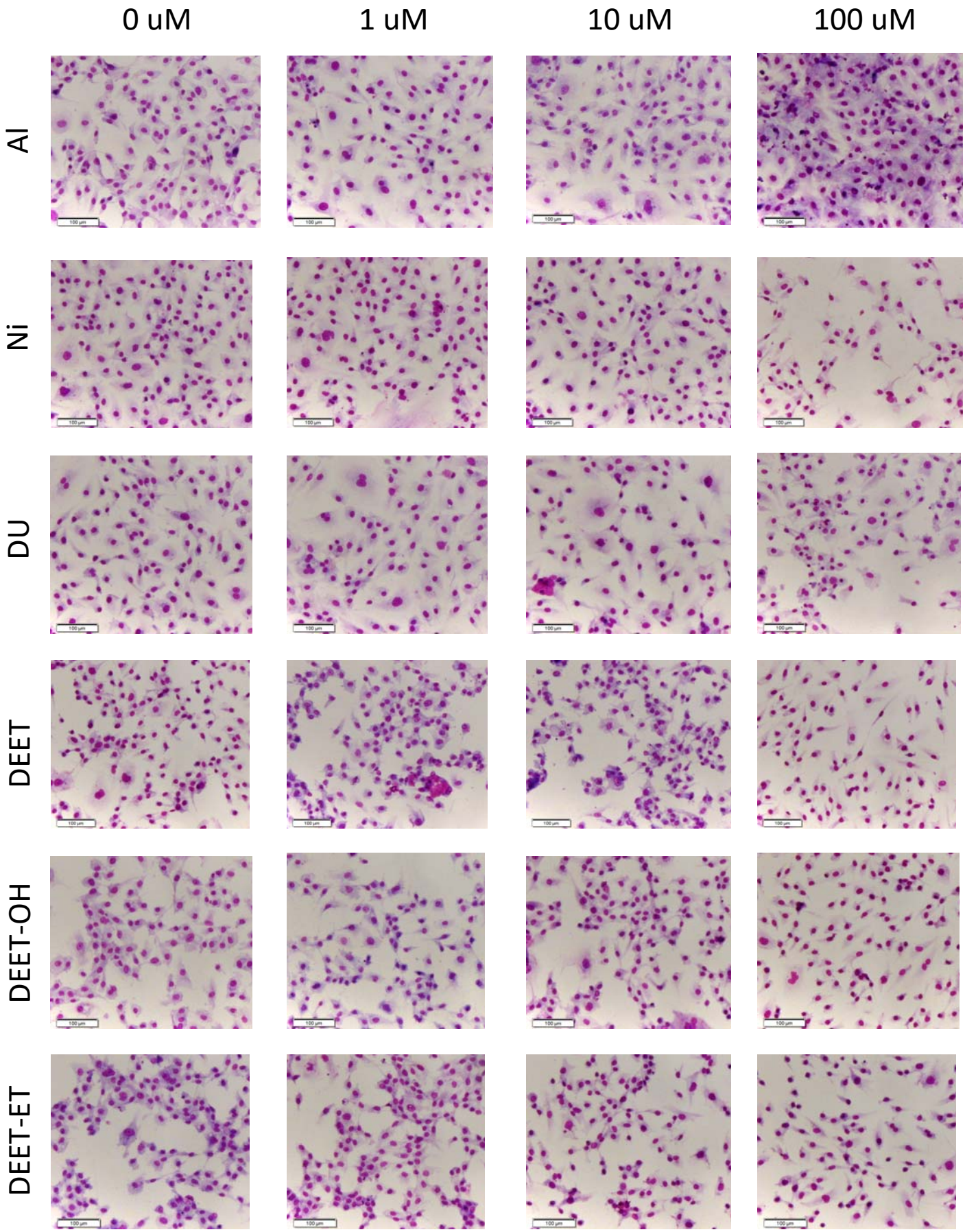


Figure 12: Human Brain Microvascular Endothelial Cells (10X)